Amperometric lytA Magnetogenosensor Applied to the Rapid and Specific Identification of *Streptococcus pneumoniae* in Clinical Samples

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ABSTRACT:

In this communication, the application of a previously developed combined asymmetric PCR-magnetic disposable amperometric genosensor to the determination of Streptococ-cus pneumoniae in clinical samples is reported. Results obtained with the amperometric magnetogenosensor assay and the gold standard protocol used (standard blood agar culture) for these tests have been compared through the analysis of 69 clinical samples and 126 agar plate cultures with suspicion of having *S. pneumoniae*. The quality of the devel-oped diagnostic test has been evaluated by constructing the corresponding Receiver Operating Characteristic (ROC) curves. Data provide a sensitivity of 90.30% and a specificity of 76.47 %, allowing unequivocal discrimination of the target bacteria from S. mitis and other closely related species. The aPCR disposable magnetogenosensor provides the results in only 5 h in comparison with the classical optochin identification test which requires up to 48 h.

Keywords: *Streptococcus pneumoniae*, clinical samples, disposable magnetogenosensor.

1. Introduction

The worldwide rise of antibiotic-resistant *Streptococcus pneumoniae* has fostered the development of methods for the accurate and rapid identification of pneumococci. Gram staining and optochin susceptibility are most frequently employed in the clinical setting al-though bacterial culture requires several days to complete [1]. In addition, the prior ad-ministration of antimicrobials reduces the possibility to accurately identify the pathogen. Immunochromatographic methods such as Binax NOW [2] have been developed in the last years for pathogen identification. Unfortunately, most of these methods, albeit providing rapid results, lack of satisfactory specificity and sensitivity. Besides, PCR amplification of pneumococcus-specific genes usually demands complicated processes and are not yet widely available in clinical laboratories.

The S. *pneumoniae* lytA gene encodes the major autolysin that has been considered unique gene to this species [3]. Although pneumococcal (and some streptococcal) temperate phages together with *Streptococcus pseudopneumoniae* harbor *lytA* homologs, the existence of characteristic signatures in this gene has allowed us to design specific oligonucleotide primers to accurately distinguish "true" pneumococci from either prophages or no pneumococcal, streptococci of the mitis group

(SMG). An amperometric magnetogenosensor has been recently developed [4]. As a proof of concept, this method has shown that it is possible to discriminate between noninoculated blood and urine samples and samples inoculated with only 10^3 CFU mL^{-1} S. *pneumoniae*.

The present communication discusses the results obtained with the *lytA* genosensor using a variety of clinical samples, and the advantages of this procedure in the rapid identification of S. *pneumoniae*.

2. Experimental Section

2.1.. Apparatus and electrodes

Amperometric measurements were carried out with a single-channel amperometric detector purchased from InBea Biosensores S. L. (Madrid, Spain). A P-Selecta (Scharlab) ultrasonic bath, a PCR cabinet Biosan DNA/RNA UV-cleaner UVC/T−M−AR, a Thermomixer MT100 constant temperature incubator shaker (Universal Labortechnik), and a P-Selecta Agimatic magnetic stirrer, were also used. Gold screen-printed electrodes (220AT, 4-mm∅, Au/SPEs) purchased from Dropsens were employed. The format of these electrodes includes a silver pseudo-reference electrode and a gold counter electrode. A neodymium magnet (AIMAN GZ) was used to control the attraction of the modified magnetic beads (MBs) to the Au/SPEs surfaces. Magnetic separation steps for incubation/washing processes were performed using a Dynal MPC-S (product No. 120.20, Dynal Biotech ASA, Norway) magnetic particle concentrator. All PCR experiments were conducted in a Mygene series Peltier thermal cycler model NGN96G.

2.2. Reagents and solutions

Streptavidin-modified MBs (10 mg mL^{-1}) (Dynabeads M-280 Streptavidin) were purchased from Dynal Biotech ASA. Streptavidin-horseradish peroxidase (HRP) polymer, ultrasensitive was purchased from Sigma and used as recommended. Stock so-lutions of streptavidin-HRP (10 μg mL^{-1}) were prepared in phosphate buffered saline (PBS; 10-mM sodium phosphate buffer, pH 7.4 containing 138 mM NaCl, and 2.7 mM KCl) supplemented with 0.05% (v/v) Tween-20 (PBST). TMB-H_2O_2-K-Blue reagent solution from Neogen in a ready-to-use reagent format (K-Blue enhanced-activity substrate, also containing H_2O_2) was also used as enzymatic substrate. The oligonucleotides used in this work were synthesized by Sigma Genosys, and are listed in Table 1.

B&W buffer solution (10 mM Tris–HCl, pH 7.5; 1 mM EDTA, 2 M NaCl) was used to prepare the biotinylated probe to be immobilized at the MBs. All chemicals used were of analytical-reagent grade, and deionized water was obtained from a Millipore Milli-Q purification system (18.2 MΩ cm).

Table 1: Oligonucleotides used in this study

Oligonucleotides	Sequence (5´→3´)
Capture probe	Biotin-TGCCGAAAACGCTTGATAC
Forward primer	TTGGGAACGGTTGCATCATG
Reverse primer	Biotin-TCGTGCGTTTTAATTCCAG

2.3. Samples and DNA isolation

A preclinical comparison between the results achieved with the amperometric magneto-genosensor assay and the gold standard protocol (standard blood agar culture) was conducted by analyzing 69 clinical samples and 126 agar plate cultures with suspicion of having *S. pneumoniae.*

Analyzed clinical isolates (sputum, pus, blood, throat swab, bronchial, nasal and tracheal aspirates, eye, conjunctive swab, alveolar washes, skin, pleural fluid, and abscess) were collected from La Paz Hospital (Ma-drid, Spain) patients.

Magnetoimmunosensor determinations were performed using DNA extracted from both kinds of samples with the UltraClean® Microbial DNA isolation kit (MO BIO, Carlsbad, CA USA) according to the manufacturer´s instructions. The extracted DNA was stored at −20 °C until use. It is worth to mention that for the analysis of the clinical samples, no other pretreatment was required previously to this DNA extraction.

The quality of the developed diagnostic test was evaluated by constructing the corre-sponding ROC curves, performing the statistical analysis with SPSS v. 17.0 software.

2.4. Asymmetric PCR amplification

The asymmetric PCR (aPCR) reaction mixture (50 μL) contained 4 μL or 2 μL (for clinical samples and agar plate cultures, respectively) of the extracted DNA, 1 μL of a 5 μM forward primer solution, 8 μL of a 5 μM reverse biotinylated primer solution, and other components following the protocol for PCR with Taq DNA polymerase. The PCR conditions were: denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min (denaturation), 60 °C for 1 min (annealing), 72 °C for 1 min (extension), and 3 min final extension. During the process, the lower concentrated forward primer acted as a limiting primer and target dsDNA fragments were produced when both primers were present in the PCR mixture. However, after the limiting primer was consumed the remaining primer (biotinylated reverse primer) continued to amplify the target DNA, which resulted in the PCR products predominantly being biotinylated ss *lytA* gene fragments.

2.5. Magnetic beads modification

For the MBs modification, 10 μL of streptavidin-coated MBs were transferred to a 1.5 mL centrifuge tube, washed twice with 100 μL of B&W buffer and resuspended in 100 μL of the same buffer containing 100 pmol of the biotinylated probe (Fig. 1). The probe was captured onto the beads for 50 min at 37 °C with gentle mixing (600 rpm). Subsequently, the probe modified MBs were washed twice with 100 μL of Tris–HCl buffer (pH 7.2) and resuspended in 100 μL of the same buffer containing the desired amount of biotinylated target (or 2 times di-luted aPCR mixture). The reaction was left to proceed for 50 min at 37 °C under stirring (600 rpm). The hybrid-attached beads were washed twice with 500 μL of Tris–HCl (pH 7.2). At this time, labeling of the target with the streptavidin-HRP polymer enzyme was achieved. Enzyme tag took place by adding 100 μL of 10 μg mL^{-1} streptavidin-HRP polymer solution prepared in PBST. The labeling procedure was left to proceed for 60 min at 37 °C with gentle mixing (600 rpm). The enzyme-tagged DNA beads assembly was then washed five times (2 min each) with 500 μL of PBST, and once more with 500 μL of PBS. MBs were finally resuspended in 45 μL of PBS.

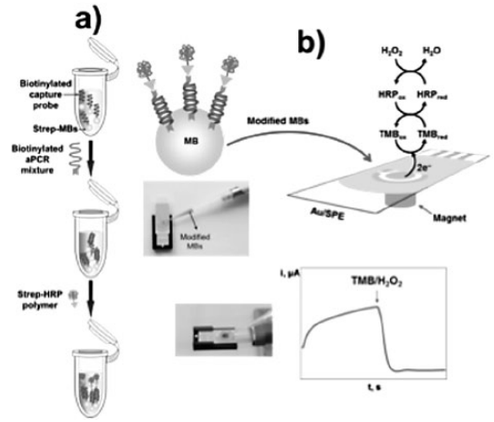

Fig. 1: Schematic representation of the MBs-modification (a) and detection (b) steps carried out.

2.6. Amperometric measurements

Amperometric measurements were carried out by placing the 45-μL drop of the modified MBs suspension onto the Au/SPEs surface, covering the three electrodes area. When a steady-state current was obtained, at an applied potential of –0.10 V vs the Ag pseudo-reference electrode, 5 μL of the TMB-H_2O_2 solution were deposited on the working electrode surface and the current signal used for analytical purposes was read 200 s afterward.

3. Results and Discussion

Amperometric measurements were performed for both, the samples and the corresponding PCR blanks, and thus, sample signals were normalized by using the blank signal. For the streptococcal cultures, samples were classified in two categories: *S. pneumoniae* vs. non-pneumoniae *Streptococcus* sp. based on the standard microbiological identification. The ratios obtained from the

amperometric measurements were compared with the identification analyses using a ROC curve analysis.

Using a cutoff value of 1.9 for the sample to blank ratio (ratio>1.9 meaning that the sample is *S. pneumoniae*) a sensitivity of 83.5 % and specificity of 72.4 % were obtained (Table 2).

Table 2: Assay results for agar plate cultures

		Microbial identification		
		+	–	
Magneto-genosensor	+	81	8	89
	–	16	21	37
		97	29	126

(+: positive sample; –: negative sample)

Concerning the clinical samples, the classification in the same two categories was based on the microbiological identification and the clinical relevance assigned by a clinical microbiologist: *S. pneumoniae* vs. S. *pneumoniae* negative samples. This last category includes sterile samples, samples containing a major non-pneumoniae *Streptococcus* sp. or other pathogens, and samples that may contain small amounts of *S. pneumoniae* but not considered clinically relevant (i.e. part of the saprophytic flora). The ROC analysis indicated that the best cutoff for the sample to blank ratio is 1.3 (ratio>1.3 meaning that the sample is clinically relevant *S. pneumoniae*). For this cutoff the sensitivity was 90.3 % and the specificity was 76.4 % (Table 3).

Table 3: Assay results for clinical samples

		Microbial identification		
		+	–	
Magneto-genosensor	+	47	4	51
	–	5	13	18
		52	17	69

(+: positive sample; –: negative sample)

It should be noted that some samples gave a positive result with the magnetogenosensor and a negative one through microbial identification. Current studies are aimed at discerning if this can be attributed to an inhibition of the bacterial growing caused by antibiotics, the lower bacterial content being only detected by the magnetogenosensor.

4. Conclusion

The results achieved in the clinical validation of this new strategy based on the use of a novel amperometric magnetogenosensor are highly promising taking into account the complexity and variability between the analyzed samples and the simplicity and the short response time of the implemented methodology. The combined a-PCR-magnetic disposable genosensor approach exhibits relevant analytical advantages with respect to other S. *pneumoniae* detection methods previously reported, allowing an unequivocal discrimination of the target bacteria from S. mitis and other closely related pneumococcal species (such as other streptococci of the mitis group, SMG) and providing results in only 5 h, in comparison with the classical optochin identification test which requires 48 h.

Acknowledgements:

This work was funded by a grant from the Ministerio de Ciencia e Innovación (IPT-2011-1337-010000). The CIBER de Enfermedades Respiratorias (CI-BERES) is an initiative of the Instituto de Salud Carlos III (ISCIII). The financial support of the Spanish Ministerio de Economía y Competitividad Research Project, CTQ2012-34238, and the AVANSENS Program from the Comunidad de Madrid (S2009PPQ-1642) are also gratefully acknowledged. We acknowledge Hospital Universitario La Paz (Madrid, Spain) for supplying us with the clinical samples.

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